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Male–female interaction and temperature variation affect pollen performance in *Citrus*

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ABSTRACT

Despite the extensive research work characterizing pollen performance in several plant species, less effort has been made to characterize it in some economically important species as *Citrus*, in which the failure of the sexual reproductive process, and subsequent parthenocarpic fruit development and seedlessness are prized characters. In this work we characterized pollen–pistil interaction in the three ancestral true-type *Citrus* species in order to determine the influence of the male and female genotypes, as well as of the temperature regime on pollen performance. Specifically, we characterized the effect of temperature on pollen grain germination *in vitro*, and on pollen tube growth *in vivo* in cut flowers and *in planta* under field temperature conditions. Results obtained showed that temperature variation has a strong effect on pollen germination, and on pollen tube kinetics and on their population size depending on the specific male–female combination. The optimum temperature for pollen germination *in vitro* was of 25 °C, while the most favorable temperature to accelerate *in vivo* pollen tube growth depended on the particular male–female interaction and ranged between 15 and 25 °C. Furthermore, temperature appears to have an effect on self-incompatibility reaction by affecting the place where pollen tubes are arrested. Overall, our results show that pollen performance is not only an inherent characteristic of the pollen genotype, but is largely dependent on the particular male–female combination and on genotype–temperature interactions.

1. Introduction

Pollen viability and performance play an important role in the sexual reproductive success of plants, finally materialized by fertilization of the ovules and the resultant formation of seeds. Extensive research work characterizing pollen performance in different plant species revealed that pollen tube growth and its likelihood to fertilize ovule depend not only on its own genotype (Snow and Spira, 1991) but also on the genotype of the pistil (Stephenson and Bertin, 1983; Willson and Burley, 1983; Herrero and Hormaza, 1996; Hormaza and Herrero, 1996, 1999; Mulcahy et al., 1996) and on the prevailing environmental conditions during flowering and pollination (Young and Stanton, 1990; Stephenson et al., 1992; Johannsson and Stephenson, 1998; Hedhly et al., 2009; Hedhly, 2011).

Characterizing pollen performance is especially relevant for some economically important genus like *Citrus*, in which failure of the sexual reproductive process resulting in parthenocarpic fruit development and seedlessness is a prized character. The importance of *Citrus* for agriculture in a world context is shown by its worldwide distribution and its large-scale production. Grown commercially in more than 100 countries around the world in tropical and sub-tropical climates (approximately 40°N and S of the equator), citrus production (including oranges, grapefruit, tangerines and mandarins, lemons and limes) has experienced continuous growth in the last decades of the twentieth century reaching a total annual production of more than 120 million tons (FAOSTAT, 2010). Despite the economic importance of *Citrus* species little research work has been done to characterize their reproductive biology. To this it could partly contribute the lack of an established boundary between species, subspecies and interspecific hybrids, and also the paucity of knowledge of their self-incompatibility reaction and parthenocarpic behavior. Although recent work (Distefano et al., 2011) shows that parthenocarpy in mandarin is related to an uncoupling of the onset of fruiting with the reproductive process.

On the one hand, the taxonomy of the genus *Citrus* is not exactly established. Recently, studies suggested that only three *Citrus* types, namely citron (*Citrus medica* L.), mandarin (*Citrus reticulata* Blanco), and pummelo (*Citrus maxima* [Burm.] Merril) constituted

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true or valid species, and that derived from inter- and intraspecific crosses between these three species are the other important *Citrus* species such as orange, lemon, lime and grapefruit (Scora, 1975, 1988; Barrett and Rhodes, 1976). The concept of the three true valid species while the other species were derived from hybridization between them is further supported from various recent studies using biochemical and molecular markers (Herrero et al., 1996; Fang and Roose, 1997; Federici et al., 1998; Nicolosi et al., 2000; Moore, 2001; Barkley et al., 2006; Deng et al., 2007).

On the other hand, little is known about the self-incompatibility and parthenocarpy behaviors of *Citrus* species, which are very important traits for fruit production and fruit quality since they result in seedless fruit. In particular, seedlessness is currently a very important feature in the evaluation of commercial citrus fruits for fresh consumption.

Also *Citrus* breeding strategies have been indeed hampered by factors associated with reproductive biology (sterility, incompatibility, nucellar embryony, juvenility). The control of self-incompatibility is thought to be gametophytic, based only on the observation of the pollen tubes growth, which usually arrest after they have covered some distance through the gynoecium (Soost, 1965). While crosses between mandarin cultivars show a clear arrest of pollen tubes in the style (Distefano et al., 2009), the incompatibility reaction has also been reported to occur along different phases of pollen tube growth through the gynoecium in different *Citrus* species (Soost, 1965; Nettancourt, 1977, 1997; Sedgley and Griffin, 1989), but more research is needed to fully characterize it as is the case for other plant families with a gametophytic self-incompatibility system such as Solanaceae (Nettancourt, 1977), Rosaceae (Sassa et al., 1993; Tao et al., 1997), Scrophulariaceae (Xue et al., 1996), Papaveraceae (Franklin-Tong et al., 1993, 1995).

In the present study, pollen–pistil interaction has been characterized in the three *Citrus* ancestral species under different temperature treatments. We first carried out a pollen germination *in vitro* experiment under five temperature regimes (10, 15, 20, 25, 30 °C), reflecting the spectrum (from cool to hot) of temperature conditions that can be found during citrus blooming season. Second, to characterize the influence of temperature in the putative site where the pollen tube growth is arrested in self-incompatible crosses we characterized pollen tube behavior in self-pollinated clementine. Third, to check for inter-species compatibilities and characterize the influence of the pistilar genotype and of temperature regimes on pollen tube growth, we carried out a diallel experiment (self- and cross-pollination) among the three ancestral species under four temperature regimes.

2. Materials and methods

2.1. Plant materials

In the present work four *Citrus* genotypes were used. Three genotypes belong to the three *Citrus* ancestral type species: the semi self-compatible 'Dancy' mandarin, 'Vozza Vozza' citron regarded as self-compatible, and the self-incompatible 'Sha Tian Yu' pummelo. The semi self-compatibility in 'Dancy' mandarin is not a barrier to perform this pollination experiment, since a half of pollen tubes grow normally in any pollination. The fourth and self-incompatible cultivar 'Comune' clementine (*C. reticulata* Blanco) was used as a known reference for self-incompatibility test (Distefano et al., 2009). Kim et al. (2011) reported the S genotype of several *Citrus* genotypes; however information about the genotypes used in this study is not evident. Trees, older than 10 years, of these four *Citrus* varieties were grown at the "Primosole" Experimental Farm of Catania University adopting standard cultural practices.

2.2. In vitro pollen germination

To obtain fresh pollen, 40 flowers were collected one day before anthesis from three trees of each genotype (clementine, mandarin, citron, pummelo). The anthers were removed and left to dehisce for 24 h at room temperature at about 25 °C. Fresh pollen was immediately used for testing *in vitro* pollen germination at five temperature regimes (10, 15, 20, 25, 30 °C) in Petri dishes on a solidified germination medium consisting of 100 g l^{-1} sucrose, 0.1 g l^{-1} H_3BO_3, 0.3 g l^{-1} $Ca(NO_3)_2$, 0.1 g l^{-1} KNO_3, and 10 g l^{-1} agarose (Mesejo et al., 2006). Pollen germination was arrested after 24 h by freezing at −20 °C. This procedure was adopted as it revealed its efficacy in preserving pollen morphology for microscopic observation (Hedhly et al., 2005a). Pollen was scored as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. For each treatment, germination was recorded in two Petri dishes by counting three complete fields, until reaching at least 100 pollen grains in each field, in each Petri dish (6 replicates).

2.3. In vivo pollen germination and pollen tube growth

To evaluate the effect of the genotype and of temperature regime on pollen behavior *in vivo*, a diallele experiment between the three ancestral species under field conditions and four temperature regimes was carried out. In the field experiment, flowers of clementine, mandarin, citron and pummelo were emasculated at balloon stage, hand-pollinated (10 flowers per treatment), bagged in cotton tissue, and sampled after 10 days. For the controlled chambers experiment, 120 flowers at balloon stage were collected randomly from three trees for each genotype, emasculated, and immediately placed in trays with soaked florist foam. Ten flowers per treatment were either self-pollinated or cross-pollinated in all possible combinations, except for clementine that was only self-pollinated. After pollination, trays were immediately placed in the controlled temperature chambers at 15 °C, 20 °C, 25 °C and 30 °C. To compare pollen performance across experiments, all *in vitro*, *in vivo*, and *in planta* pollinations were performed with the same batch of pollen. Self- and cross-pollinated flowers of the four species were collected three days after pollination, fixed in a FAA (formalin, glacial acetic acid, 70% ethanol, 1:1:18, v/v; Johansen, 1940), and maintained at 4 °C until microscopic observation.

2.4. Microscopic observations

Pollen grain germination and pollen tube growth were monitored on squash preparations. Pistils fixed in FAA were washed three times in water, 1 h each, and left in 5% sodium sulfite solution overnight (Jefferies and Belcher, 1974). Pistils were, then, softened in 5% sodium sulfite solution in a microwave for 45 s. Before squashing the preparations, the ovary was cut from the stigma-style, and both parts were further cut longitudinally and split into two parts. Following the staining procedure and squashing with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens and Esser, 1957), the preparations were observed under a fluorescence microscope (Leica DM 2500 of Leica Microsystems GmbH using I3 filter excitation 450–490 nm, Wetzlar, Germany). Pollen tube growth was recorded as the length of the longest pollen tube in the stigma-style, measured in 10 pistils/genotype. The number of pollen tubes reaching the base of the style was also recorded.

2.5. Statistical analyses

For statistical analysis, to normalize data and homogenize variances, all count data were square-root-transformed before carrying out the analyses of variance. In the case of pollen germination *in vitro*, the removal of 10 °C observations allowed a better